Structure-Function Analysis of a Broad Specificity *Populus trichocarpa* Endo-β-glucanase Reveals an Evolutionary Link between Bacterial Licheninases and Plant *XTH* Gene Products*[s]

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Background: The evolution of the plant xyloglucan endotransglycosylase/hydrolase (*XTH*) genes in glycoside hydrolase family 16 (GH16) is enigmatic.
Results: A unique, mixed function endo(xylo)glucanase from black cottonwood has been biochemically and structurally characterized.
Conclusion: This enzyme is an important link between extant bacterial endoglucanases and plant *XTH* gene products.
Significance: New insights into the molecular evolution of *XTH* gene products and further unification of GH16 enzymes have been gained.

The large xyloglucan endotransglycosylase/hydrolase (*XTH*) gene family continues to be the focus of much attention in studies of plant cell wall morphogenesis due to the unique catalytic functions of the enzymes it encodes. The *XTH* gene products compose a subfamily of glycoside hydrolase family 16 (GH16), which also comprises a broad range of microbial endoglucanases and endogalactanases, as well as yeast cell wall chitin/β-glucan transglycosylases. Previous whole-family phylogenetic analyses have suggested that the closest relatives to the *XTH* gene products are the bacterial licheninases (EC 3.2.1.73), which specifically hydrolyze linear mixed linkage $\beta(1\rightarrow3)/\beta(1\rightarrow4)$-glucans. In addition to their specificity for the highly branched xyloglucan polysaccharide, *XTH* gene products are distinguished from the licheninases and other GH16 enzyme subfamilies by significant active site loop alterations and a large C-terminal extension. Given these differences, the molecular evolution of the *XTH* gene products in GH16 has remained enigmatic. Here, we present the biochemical and structural analysis of a unique, mixed function endoglucanase from black cottonwood (*Populus trichocarpa*), which reveals a small, newly recognized subfamily of GH16 members intermediate between the bacterial licheninases and plant *XTH* gene products. We postulate that this clade comprises an important link in the evolution of the large plant *XTH* gene families from a putative microbial ancestor. As such, this analysis provides new insights into the diversification of GH16 and further unites the apparently disparate members of this important family of proteins.

Plant cell walls are complex biocomposites, comprising semicrystalline and amorphous polysaccharides, polyphenolics, structural proteins, and inorganics, which are of biological, ecological, and industrial importance (1–3). In addition to forming the basis of the agricultural and forest products industries, plant cell walls represent a vast sink in the global carbon cycle. Thus, understanding molecular mechanisms of plant cell wall biosynthesis and morphogenesis is fundamentally important (4).

Depending on the cell type and developmental stage, carbohydrates typically compose at least three-fourths of the plant cell wall dry weight (1, 5). Semicrystalline cellulose is the main load-bearing polymer embedded in a hydrated network of matrix glycans. A diversity of primary plant cell wall polysaccharide compositions can be observed (6). For example, the walls of dicots and non-commeneloid monocots contain xyloglucans as the primary matrix polysaccharide, whereas the grasses utilize mixed linkage $\beta(1\rightarrow3)/\beta(1\rightarrow4)$-glucans in this role (1, 5). Due to this predominance of structural carbohydrates, the biosynthesis and enzyme-catalyzed rearrangement of polysaccharides underpin contemporary cell wall models (4). Indeed, plant genomes encode large repertoires of glycosyltransferases, glycoside hydrolases, and transglycosidases, often in large, multigene families (7–10).

In the context of postbiosynthetic plant cell wall remodeling, there is a sustained interest in the xyloglucan endotransglyco-

sylase/hydrolase (*XTH*)[2] gene family. This is due to the remarkable ability of some gene products to catalyze matrix polysaccharide rearrangement with essentially no chain hydrolysis (XET activity, EC 2.4.1.207). On the other hand, a limited number of *XTH* genes encode predominant hydrolases (XEH, EC 3.2.1.151) that operate in germinating seeds, ripening fruit, or expanding tissues (11, 12). Notably, contemporary enzyme structure-function analyses have led to a refined phylogenetic delineation of the disparate transglycosylating and hydrolytic activities individual *XTH* gene products, which number in the range of ~20–60 in diverse plants (11, 13).

However, the molecular evolution of the *XTH* gene products in the larger context of glycoside hydrolase family 16 (GH16) (14) remains enigmatic. In addition to the plant *XTH* gene products, GH16 counts a broad range of microbial endoglucananases and endogalactanases among its functionally characterized members, spanning those active on terrestrial and marine polysaccharides to yeast cell wall chitin/β-glucan transglycosylases (13). Despite significant sequence divergence, all GH16 enzymes are predicted to share both a common overall β-jellyroll protein fold and the canonical retaining GH catalytic mechanism (15–17).

Previous whole-family phylogenetic analyses have suggested that the closest relatives to the *XTH* gene products are the bacterial licheninases (EC 3.2.1.73), which specifically hydrolyze $\beta(1\rightarrow4)$ linkages in mixed linkage $\beta(1\rightarrow3)/\beta(1\rightarrow4)$-glucans (15). *XTH* gene products are, however, distinguished from the licheninases and all other GH16 enzyme classes by significant differences in their common β-jellyroll protein structure. These differences include major loop alterations and a unique C-terminal extension, in addition to a singular specificity for a highly branched polysaccharide substrate (13). Exactly how the *XTH* gene products may have arisen from licheninases has heretofore remained unclear.

Here, we present the biochemical and structural analysis of an unusual, mixed function endoglucanase from black cottonwood (*Populus trichocarpa*). This research reveals a small clade of GH16 members intermediate between the bacterial licheninases and plant *XTH* gene products. We postulate that this clade comprises an important link in the evolution of the large plant *XTH* gene families from a putative microbial ancestor. As such, this analysis provides new insights into the diversification of GH16 and further unites the apparently disparate members of this important family of proteins.

EXPERIMENTAL PROCEDURES

Substrates—Xyloglucan, barley mixed linkage glucan (medium viscosity), Icelandic moss lichenan, laminarin, lupin β-galactan, wheat arabinoxylan, carboxymethyl cellulose (CMC), cellooligosaccharides, and mixed linkage glucooligosaccharides were from Megazyme. Hydroxyethyl cellulose was from Fluka. Xyloglucooligosaccharides were prepared as described previously (18).

Bioinformatic and Phylogenetic Analyses—The EG16-like enzymes were found using *Pt*EG16 as the query with BLASTp at the Phytozome Web site (October 2012). The Charophyta sequence CHARA2 was manually transcribed from Ref. 19, and an expressed sequence tag from *Coleochaete nitellarum* was obtained from GenBank[TM] (accession number HO204633). Possible localization and post-translational modifications were analyzed by SignalP (20), ChloroP (21), LipoP (22), and GPP (23). These sequences were also included in the phylogeny: *XTH* gene products from rice, *Oryza sativa* (*Os*XTHs), and *Arabidopsis* (*At*XTHs); Group III-A sequences from Ref. 11; and selected Group IIIA sequences from TIGR (24). Two *Bacillus* licheninases (PDB codes 1gbg and 1u0a) were included to root the tree. Sequences were aligned using MUSCLE (25), and the alignment was edited manually in Bioedit (26), guided by the available three-dimensional structures. Maximum likelihood and Bayesian phylogenies were built using PhyML 3.0 (27) and MrBayes 3.1.2 (28), respectively. In PhyML, the reliability of nodes was tested by 100 resamplings. In MrBayes, 2×10^6 generations were run with a sample frequency of 100. Blosum62 was used as the amino acid substitution matrix in both PhyML and MrBayes. Trees were drawn with MEGA5 (29).

Protein Expression and Purification—*Pt*EG16 cDNA (originally codon-optimized for *Pichia pastoris* expression; GENEART AG) was cloned into Expresso N-His vector with an N-terminal His$_6$ tag (Lucigen). *Pt*EG16 was produced in Hl control *Escherichia coli* BL21 (DE3) cells (Lucigen) grown in Terrific Broth at 37 °C at 200 rpm to an A_{600} of 0.8 and then induced by the addition of isopropyl β-D-galactopyranose to a final concentration of 0.5 mM. During the induction phase, the culture was kept at 25 °C overnight. Cells were then collected by centrifugation at $4800 \times g$ for 10 min at 4 °C. The cells were resuspended in buffer A (25 mM sodium phosphate, 0.5 M NaCl, and 25 mM imidazole, pH 7.5) and ultrasonicated to liberate cytoplasmic proteins. The supernatant was collected by centrifugation at $24,700 \times g$ for 15 min at 4 °C and passed through a 5-ml HisTrap FF Crude column (GE Healthcare). The protein was eluted using a 10-column volume-long linear gradient with buffer B (25 mM sodium phosphate, 0.5 M NaCl, and 0.5 M imidazole, pH 7.5). Fractions containing *Pt*EG16 were pooled, and DTT was added to a final concentration of 2 mM. After incubation at room temperature for 2 h, the sample was purified further using an XK 16/100 Superdex 75 column (GE Healthcare), pre-equilibrated with 20 mM MOPS, 1 mM tris(2-carboxyethyl)phosphine, pH 7.5. Monomeric *Pt*EG16 fractions were pooled, concentrated using Vivaspin20 5 kDa (PES) centrifugal concentrators, and stored at 4 °C.

*Pt*EG16 enriched in ^{15}N for NMR experiments was produced using *E. coli* cells grown at 37 °C at 200 rpm to an A_{600} of 1 in LB, centrifuged at $3000 \times g$ for 10 min, and then gently resuspended in M9 medium containing 1 g/liter ^{15}NH$_4$Cl and 0.5 mM isopropyl β-D-galactopyranose. The induced culture was kept at 16 °C for 30 h before the cells were collected by centrifugation at $4800 \times g$ for 10 min at 4 °C. The ^{15}N-labeled *Pt*EG16 was treated and purified as described above for the non-labeled enzyme.

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[2] The abbreviations used are: XTH, xyloglucan endotransglycosylase/hydrolase; XET, xyloglucan:xyloglucosyl transferase; XEH, xyloglucan-specific endo-β-1,4-glucanase; GH, glycoside hydrolase; GH16, glycoside hydrolase family 16; CMC, carboxymethyl cellulose; HSQC, heteronuclear single quantum coherence; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; TROSY, transverse relaxation optimized spectroscopy; PDB, Protein Data Bank.

Triple-labeled *Pt*EG16-1 (^{2}D, ^{13}C, and ^{15}N) was produced by first inoculating 600 ml of M9 medium with 3 ml of overnight LB culture (H$_2$O) and kept at 37 °C at 200 rpm until an A_{600} of 0.5. The cells were gently pelleted by centrifugation at $1000 \times g$ for 8 min and resuspended in 600 ml of M9 medium containing 1 g/liter ^{15}NH$_4$Cl, 2.5 g/liter ^{2}H$_7$/^{13}C$_6$-glucose in D$_2$O. At A_{600} 0.65, the culture was moved to 25 °C and induced after 20 min with isopropyl β-D-galactopyranose at a final concentration of 1 mM. After overnight induction, the cells were harvested at $4800 \times g$ for 15 min at 4 °C. Cells were sonicated and initially purified on a 5-ml HisTrap FF crude column as for the unlabeled protein. After initial purification, the buffer was exchanged to 25 mM MOPS, 2 mM tris(2-carboxyethyl)phosphine, pH 7.5. To reintroduce ^{1}H on the nitrogens, the triple-labeled *Pt*EG16 was denatured by 1:20 dilution in 8 M urea, 25 mM Tris, pH 7.5, with 2 mM DTT prepared in H$_2$O. On-column refolding was performed essentially as described (30), with the exception that 2 mM DTT was added to all buffers. The refolded triple-labeled *Pt*EG16 was buffer-exchanged to 20 mM MOPS, 2 mM tris(2-carboxyethyl)phosphine, pH 7.5, before NMR experiments.

Activity on Polysaccharides Detected by Gel Permeation Chromatography—Assays in a total volume of 150 µl, containing 2.5 g/liter polysaccharide, 30 mM NH$_4$OAc, 0.25 mM DTT, and 24 µg/ml *Pt*EG16, were incubated for 0, 6, 20, 60, 180, and 480 min and overnight at 22 °C. The reactions were stopped by heating to 95 °C for 10 min. The samples were then lyophilized and dissolved in 200 µl of DMSO before analysis by gel permeation chromatography, as described previously (18). Control assays without enzyme were also monitored to detect background hydrolysis. Carboxymethyl cellulose and Konjac glucomannan (Megazyme) were insoluble in DMSO and were therefore analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

HPAEC-PAD—Samples were analyzed on a Dionex ICS-5000 HPAEC-PAD system using a Dionex CarboPac PA-200 column. Four different programs (supplemental Table S1) were used, depending on the sample. Program A was used to identify the limit digestion products of lichenan and mixed linkage glucans *versus* standard samples of cello-oligosaccharides (degree of polymerization 1–6) and three mixed linkage glucan tetraoses: MLGA, G3G4G4G; MLGB, G4G4G3G; and MLGC, G4G3G4G. Program B (18) was used to analyze the limit digestion products of all other polysaccharides. Potential transglycosylation products at maximal substrate concentrations (10-min reactions) were analyzed by Program C (for cello-oliogsaccharides and mixed linkage glucan oligosaccharides) and Program D (for XXXGXXXG).

Quantitative Kinetic Analysis of PtEG16 Activity on Oligosaccharides—Kinetic quantitation under initial rate conditions was determined on cello-oligosaccharides (degree of polymerization 2–6) and the xylogluco-oligosaccharide XXXGXXXG using HPAEC-PAD. The pH optimum of the enzyme was determined by incubation of 30 nM PtEG16 with 400 µM cellohexaose and 50 mM buffer (sodium citrate for pH 3–6.5 and MOPS for pH 6.5–8.1) in a final volume of 40 µl. All subsequent assays with oligosaccharide substrates were per-

formed in 40-µl reactions buffered with 25 mM sodium citrate, pH 5.25. In all cases, assays were maintained at 22 °C and terminated by the addition of 10 µl of 1 M NaOH prior to analysis using Program A (supplemental Table S1). Peak areas were quantified by integration and converted to molar amounts based on standard curves in the range 1–50 µM.

Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) Analysis—Oligosaccharide products were analyzed by MALDI-TOF mass spectrometry in positive ion mode on an LT3 Plus mass spectrometer (SAI Ltd.) operated by the MALDI Mainframe 2, MALDI Control software (version 1.03.51, SAI Ltd.). Samples were supplemented with NaCl to a final concentration of 20 mM, and 2,5-dihydroxybenzoic acid (10 g/liter in water) was used as the matrix.

NMR Spectroscopy—Resonance assignments for the main chain ^{1}H^N, ^{15}N, and ^{13}C nuclei in the 220-residue carbon-perdeuterated ^{13}C/^{15}N-labeled His$_6$-tagged *Pt*EG16 were obtained using standard pulse sequences recorded at 25 °C on a Bruker Avance III 600-MHz spectrometer equipped with a TCI CryoProbe. The resulting ^{15}N TROSY HSQC (31–35), HNCACB (36, 37), HN(CO)CACB (36, 37), HNCO (38), and HN(CA)CO (37) spectra were processed with NMRPipe (39) and analyzed with Sparky (40). The ^{1}H and ^{15}N dimensions were corrected by +46 Hz and −46 Hz, respectively, to adjust for the TROSY shift. Chemical shift-based secondary structural predictions were obtained using the programs MICS (41), TALOS+ (42), and δ2D (43). Referencing and adjustment of the carbon shifts for deuterium isotope effects were done using TALOS+ (42).

RESULTS

Bioinformatics Analysis Reveals a Unique XTH-like Gene Product of the P. trichocarpa Genome—Using amino acid sequence alignments, we identified a unique protein encoded by the *P. trichocarpa* genome (genome version 2.2, locus POPTR_0002s15460, also known as eugene3.00021425 or *PtXTH8* (8)). This protein clearly lacked the C-terminal extension diagnostic of GH16 *XTH* gene products (44, 45) (Pfam *XET_C* (PF06955) (46)) but otherwise showed significant sequence similarity to known XETs and XEHs (Fig. 1). A BLASTp search of this sequence against predicted proteomes available at Phytozome revealed that the genomes of many embryophytes encode one or more homologous proteins with Expect (E) values less than 10^{-80}. In comparison, proteins possessing the *XTH*-specific C-terminal extension typically had E values above 10^{-35} in this analysis (data not shown), which further strengthened the conclusion that the POPTR_0002s15460 gene product (hereafter renamed as "*P. trichocarpa* endoglucanase 16" (*Pt*EG16) in accordance with biochemical data; see below) and its homologs indeed composed a separate clade. A multiple-protein sequence alignment of these homologs from diverse plants is shown in supplemental Fig. S1. Notably, none has predicted signal peptides for apoplast, organelle, or membrane localization, according to SignalP (20), ChloroP (21), and LipoP (22).

We then performed independent Bayesian and maximum likelihood phylogenetic analyses with these sequences and all 33 *Arabidopsis thaliana* and 29 *Oryzae sativa XTH* gene products (9) as representative dicot and monocot sequences, respec-

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                                          →                →          TT      →  ℓℓℓ→  TT              →             TT
Licheninase                                   ℓℓℓℓ          →                ℓℓℓ    →ℓℓℓ      →
PttXET16-34
TmNXG1
                 1          10            20            30            40            50            60
Licheninase      ........QTGGSFFEPFNSYNSGTWEKAD GYSNGGVFNCTW RANNVNFTNDG.KLKLGLTSSAYNKFDC
PtEG16           .........MADPAIHHETQPINQIAIDY............TPEACTHCPESNSITLTYD.....HRGG
PttXET16-34      .........AFAALRKPVDVAFGRNYVPTW............AFDHIKYFNGGNEIQLHLD.....KYTG
TmNXG1           AYVQGPPSPGYYPSSQITSLGFDQGYTNLW............GPQHQRVDQG..SLTIWLD.....STSG
                                                         Licheninase loop

                 →→        →→→→         TT  →      ℓℓℓ          →      TT      TT    →   TT
Licheninase
PttXET16-34      →→        →→→→         TT  →                   →      TT            →   TT
TmNXG1                                  TT                             TT
                 70         80          90          100          110          120
Licheninase      AEYRSTNIYGYGLYEVSMKPAK..NTGIVSSFFTYTGPAHGTQW DEIDIEFLG KD...TTK VQFNYYTNG
PtEG16           ARWRSTTRFLYGTFSSLIQCPKGNTSGLNFNIYLSSLEGD.KSQ DEIDFEFLG KD...KTI VQTNYYASG
PttXET16-34      TGFQSKGSYLFGHFSMQMKLVPGDSAGTVTAFYLSSQN...SEH DEIDFEFLG NRTGQPYIL QTNVFTGG
TmNXG1           SGFKSINRYRSGYFGANIKLQSGYTAGVITSFYLSNNQDYPGKH DEIDIEFLG TIPGKPYT LQTNVFIEG
                                                             * * *

Licheninase                          ℓℓℓ       →    TT   →  TT                      →→
PttXET16-34      →→                             →        →      TT       ℓℓℓ        →→
TmNXG1                               TT                         TT             ℓℓℓ
                 130         140          150          160          170          180
Licheninase      V GG.....H EKVIS. LGF DASKG FHT YAFD WQPGY. IKWYVDG VLKHTATA NIPST.......PGKIMMN
PtEG16           T GN.....R EEIHD. IGF DCSDA FHE YVIK WCPNF. IEWLIDG KVVRKVEK REGEG..FPE.KPMFLYAS
PttXET16-34      K D.....R PQRIY. IWF DPTKE FHY SVL WNMYM. TVFLVDD VPIRVFKN CKDLGVKFPFNQ PMKIYSS
TmNXG1           SGD YNIIG REMRIH. LWE DPTQD YHN YAIY WTPSE. ILFFVDD VPIRR YPRKSDAT..FPL. RELWVYGS
                               XEH loop

Licheninase      →                ℓℓℓℓ        ℓℓℓ            →→→→→→→         TTT       ℓℓℓ ℓℓℓ    ℓℓℓℓℓℓℓℓ
PttXET16-34      →                ℓℓℓℓ        →→→            →→→→→→→    TT  TT          ℓℓℓ ℓℓℓ    ℓℓℓℓℓℓℓℓ
TmNXG1           →                →→→         →→→                        →→→
                 190         200          210
Licheninase      LWNGT..GVDDWLGSYNG.ANPLYAEYDWVKYTSN..........................................
PtEG16           IWDASYICDATWTGPYMGCDAPYVCLYKDICVPVGTAVECSCDS.................................
PttXET16-34      LWNADDWATRGGLEKTDWSKAPFIASYRSFHIDGCEA SVEAKFCATQGARWWDQKEFQDLDAFQYRRLSW
TmNXG1           VWDASSWATENGKYKADYRYQPFVGKYEDFKLGSCTV EAASS.CNPASVSP.....YGQLSQQQVAAMEW
                                                         XET/XEH C-terminal extension

PttXET16-34      ℓℓℓℓ    →  ℓℓℓ              ℓℓℓℓ
TmNXG1           ℓℓℓℓℓ    →  ℓℓℓ TT   ℓℓℓ TT    ℓℓℓℓ

Licheninase      ...........................................
PtEG16           ...........................................
PttXET16-34      VRQKYTIYNYCTDRSRYPSMPPECKRDRDI
TmNXG1           VQKNYMVYNYCDDPTRDHTLTPEC......
                 XET/XEH C-terminal extension
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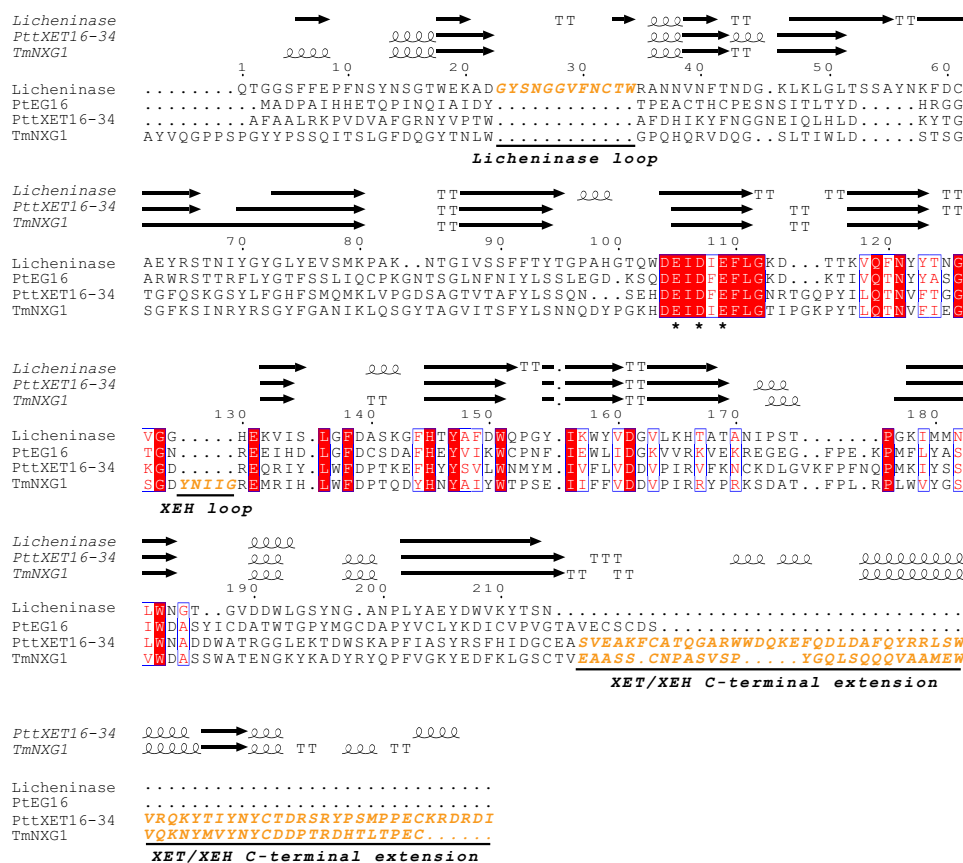
FIGURE 1. **Amino acid sequence alignment of *Paenibacillus macerans* licheninase (PDB code 1u0a), *P. trichocarpa* endoglucanase 16 (*Pt*EG16, POPTR_0002s15460), *P. tremula x tremuloides* xyloglucan endotransglycosylase 16-34 (*Ptt*XET16-34, PDB code 1umz), and *Tropaeolum majus* xyloglucan endotransglycosylase (*Tm*NXG1, PDB code 2uwa) of GH16.** The alignment was performed with MUSCLE (25), and secondary structure elements (strands (*arrows*), helices (*spirals*), and turns (*T*)) from the corresponding crystal structures were added with ESPript (83) (protein names are in *italic type*). Conserved catalytic residues are marked with *asterisks*.

tively. Crystallographically characterized XETs and XEHs (11, 45) as well as select phylogenetic Group III-A *XTH* gene products were also included in this analysis. The sequences of two *Bacillus* GH16 licheninases with known tertiary structures (47, 48) were used as more distant outliers to root the phylogenetic trees. In this analysis, only the common GH16 domain was used (*i.e.* the unique *XTH* gene product C terminus was excluded from the alignments).

Notably, the inclusion of the *Pt*EG16-like sequences in Bayesian and maximum likelihood phylogenies did not alter the overall relationships of the true *XTH* gene products observed previously (9, 11, 49, 50). Rather, *Pt*EG16 and homologs formed a major distinct clade with strong statistical support (Fig. 2*A*). Relative tree branch lengths further indicated that this clade is intermediate between the true *XTH* gene products and their presumed bacterial ancestors, the licheninases (15). Monocot and dicot sequences are clearly separated in the tree, thus indicating a potential divergent evolution of *Pt*EG16-like proteins in these two groups of flowering plants. A similar distinction can be seen among the hydrolytic Group III-A *XTH* gene products, where monocot, dicot, and non-angiosperm sequences cluster separately (Fig. 2*A*).

A Census of PtEG16-like Proteins in Plants—After establishing that the *Pt*EG16-like proteins formed a unique phylogenetic clade, we were then able to examine their presence or absence

as a class within predicted plant proteomes available via Phytozome (51), thereby extending our previous census of Group I/II, III-A, and III-B *XTH* gene products (13). Although plants generally maintain large *XTH* gene families with tens of members, the *Pt*EG16-like proteins occur in very limited numbers and are not found across all lineages (Fig. 2*B*). Notably, members of the clade containing *Pt*EG16 are not found in the two chlorophytic algal genomes presently available; these genomes also lack *XTH* or licheninase-encoding genes. In the current absence of whole genome sequences, we were able to identify two partial expressed sequence tag sequences from charophycean green algae (*Chara vulgaris* CHARA2 (19) and *C. nitellarum* GenBank[TM] accession number HO204633 (52)) whose proteins appear to exhibit greater resemblance to *Pt*EG16 than to true *XTH* gene products (supplemental Fig. S1 and Fig. 2*A*). These observations imply that *Pt*EG16-like proteins may have first arisen in the charophytes.

Regardless, *Pt*EG16-like proteins are conclusively found in an extant member of one of the oldest lineages of land plants, the moss *Physcomitrella patens*, which has three homologs. The lycophyte *Selaginella moellendorfii*, an early tracheophyte, contains only one *Pt*EG16 homolog. Among the angiosperms, all available grass *in silico* proteomes (order Poales) likewise harbor one *Pt*EG16 homolog each. The grass *Pt*EG16 homologs are, however, distinguished by longer C termini, which none-

A.

EG16-like

Licheninases

Group III-A

Group III-B

Group I/II

B.

theless do not have significant similarity to the diagnostic C-terminal extensions of true *XTH* gene products (supplemental Fig. S1). Interestingly, *Pt*EG16 homologs are not consistently found in dicotyledonous genomes. For example, they are missing in *Medicago truncatula* but are found in *Glycine max*; both are species within the Fabales. *Pt*EG16 homologs are notably absent from the predicted proteomes of two *Arabidopsis* model species as well as other important dicots (Fig. 2*B*).

Biochemical Analysis Defines PtEG16 as a Broad Specificity Endo(xylo)glucanase—To elucidate the function of the *P. trichocarpa* POPTR_0002s15460 gene product, the protein was produced recombinantly in *E. coli*. Purification to homogeneity was achieved by immobilized metal ion affinity chromatography and subsequent gel filtration chromatography. The protein was then assayed for activity against typical polysaccharide substrates for the most closely related GH16 enzymes (*i.e.* the mixed linkage $\beta(1\rightarrow3)/\beta(1\rightarrow4)$-glucans from barley and Icelandic moss and the highly branched galactoxyloglucan from tamarind seed, as well as wheat arabinoxylan, lupin β-galactan, laminarin (a mixed linkage $\beta(1\rightarrow3)/\beta(1\rightarrow6)$-glucan), hydroxyethyl cellulose, and CMC).

Initial activity screening of purified recombinant *Pt*EG16 against these polysaccharides was unsuccessful. Closer inspection of the amino acid sequence and a tertiary structure homology model (see below) revealed that the 25-kDa protein had 11 cysteine residues, 5–7 of which were likely to be surface-exposed. Subsequently, we assayed the protein in the presence of DTT and analyzed polysaccharide depolymerization by gel permeation chromatography; the presence of the reducing agent effectively precluded the use of carbohydrate reducing end assays, such as the BCA assay (53) or the Nelson-Somogyi assay (54). In this way, the ability of *Pt*EG16 to efficiently cleave barley β-glucan, Icelandic moss lichenan, and tamarind seed xyloglucan was clearly revealed (Fig. 3). In contrast, no change in polysaccharide molecular mass was observed under identical conditions for wheat arabinoxylan, lupin galactan, or laminarin, even after extensive incubation (24 h with 1 μM *Pt*EG16; data not shown).

*Pt*EG16 was also active on the artificial, soluble cellulose derivatives hydroxyethyl cellulose and CMC. The activity of *Pt*EG16 on hydroxyethyl cellulose was complex; an initial rapid molecular mass shift was observed, followed by a much slower phase of limited degradation, which may indicate that only a few sites on this modified cellulose were susceptible to *Pt*EG16-1 (supplemental Fig. S2). Analysis of CMC degradation was complicated by the observation that this polymer, as obtained from the supplier, was insoluble in the gel permeation chromatography eluent (100% DMSO). An overnight digest sample of CMC was, however, soluble in this solvent, which was indicative of reduction of the polymer molecular mass. HPAEC-PAD (data not shown) and MALDI-TOF MS (supple-mental Fig. S3) analysis of this reaction subsequently revealed the presence of short cello-oligosaccharides and carboxy-methylated derivatives.

Limit Digestion Products from Mixed Linkage Glucans—Further analysis of the products from overnight digestion of mixed linkage $\beta(1\rightarrow3)/\beta(1\rightarrow4)$-glucans and (galacto)xyloglucan using HPAEC-PAD and MALDI-TOF MS confirmed that *Pt*EG16 is indeed a broad specificity endo-$\beta(1\rightarrow4)$-glucanase. Upon extended incubation with the enzyme, barley β-glucan and laminarin were reduced to glucose, disaccharides (cellobiose/laminaribiose), and tetrasaccharides as predominant products (supplemental Fig. S4). Closer analysis of the tetrasaccharide peak resulting from either substrate (supplemental Fig. S4) indicated that this represented a single isomer with a retention time distinct from the available G4G4G3G, G3G4G4G, and G4G3G4G tetrasaccharide standards (where "G" represents Glc and the Arabic numeral represents a corresponding $\beta(1\rightarrow3)$ or $\beta(1\rightarrow4)$ glycosidic linkage). Considering the native polysaccharide structure (55), the other possible tetrasaccharide products from the mixed linkage glucans are cellotetraose (G4G4G4G) and G3G4G3G. Because the retention time of cellotetraose is shorter than the available mixed linkage tetrasaccharide standards under these HPAEC conditions (data not shown), we tentatively assign the observed product peak as G3G4G3G.

When explicitly tested for activity on the three commercially available mixed linkage tetraoses, *Pt*EG16 only hydrolyzes the central $\beta(1\rightarrow4)$ linkage of G4G4G3G, thereby yielding G4G and G3G. This suggests that the possible modes for hydrolysis of the $\beta(1\rightarrow4)$ glucosidic bonds in G3G4 ↓ G4 ↓ G and G4 ↓ G3G4 ↓ G (potential cleavage sites indicated by arrows) are catalytically insignificant for these short substrates due to geometrical requirements (*i.e.* rejection of Glc$\beta(1\rightarrow3)$ in subsites −1, −2, and −3) and/or a lack of a sufficient number of glucosyl units bound in the positive or negative enzyme subsites (see Ref. 56 for GH subsite nomenclature). Nonetheless, the production of G3G4G3G from mixed linkage glucans, as suggested above, would require acceptance of Glc$\beta(1\rightarrow3)$ units in subsite −2. This implies that extended binding of polysaccharides in the active-site cleft may overcome such limitations. Regardless, the mode of action of *Pt*EG16 on mixed linkage glucans appears distinct from that of archetypical GH16 licheninases, which explicitly require a $\beta(1\rightarrow3)$ linkage to span the −2 and −1 subsites to yield limit digestion products of the series G3G, G4G3G, G4G4G3G, and G4G4G4G3G (16).

Limit Digestion Products from Galactoxyloglucan—Despite a long incubation time, the hydrolysis of tamarind seed galactoxyloglucan by *Pt*EG16 did not go to the same level of completion (supplemental Fig. S5) previously observed for plant and microbial XEHs (12, 18). In addition to the mixture of oligosaccharides expected from cleavage at the unbranched $\beta(1\rightarrow4)$-

FIGURE 2. **Phylogenetic relationship of *Pt*EG16-like proteins in GH16 and census among plant proteomes.** *A*, phylogenetic relationship of EG16s to *XTH* gene products and licheninases. *XTH* gene product groups are according to Ref. 11. The confidence at each node in the Bayesian tree is given in posterior probabilities from the Bayesian analysis multiplied by 100. At selected nodes, the bootstrap values from maximum likelihood calculations are indicated in *parentheses*. *A. thaliana*, *O. sativa*, and *P. patens* identifiers are according to Refs. 9 and 82). Specific EG16 sequence identifiers can be found in supplemental Table 2, and gymnosperm transcripts in Group III-A are denoted by their accession number at TIGR (24). *B*, occurrence of *XTH* Group, Group II, Group III-A, Group III-B, and *Pt*EG16-like gene products (*hatched*) in selected plant *in silico* proteomes. The number of *Pt*EG16-like members in charophycean green algal proteomes is presently unknown in the absence of genome data; however, corresponding transcripts have been tentatively identified (see "Results").
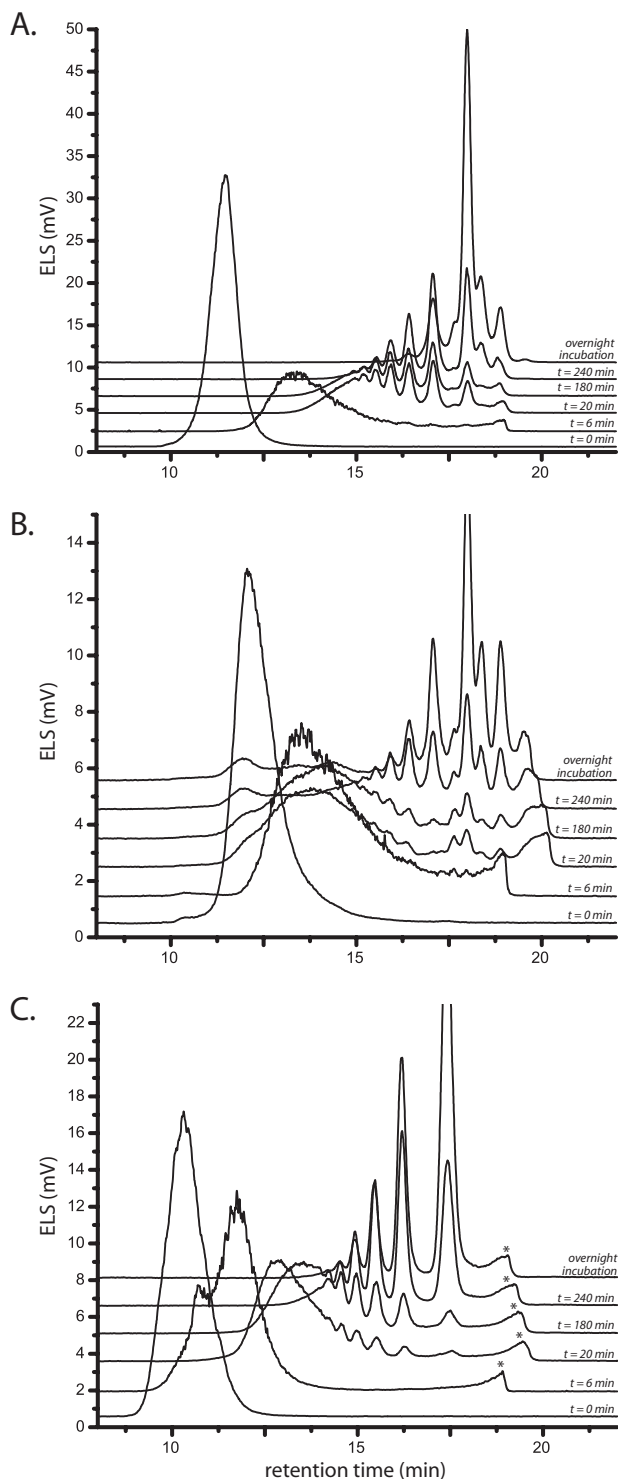
FIGURE 3. **Time-dependent endohydrolytic cleavage of polysaccharides by *Pt*EG16.** The action of *Pt*EG16 on barley β-glucan (*A*), Icelandic moss lichenin (*B*), and tamarind seed galactoxyloglucan (*C*) was analyzed using high performance size exclusion chromatography with evaporative light-scattering detection (*ELS*). The *t* = 0 trace corresponds to that of the corresponding polysaccharide before the addition of the enzyme. Compositional analyses of the oligosaccharide mixtures resulting from overnight incubation are shown in supplemental Figs. S3–S5. Peaks marked with an *asterisk* arise from low components the enzyme preparation (*e.g.* buffer and salts).

linked glucosyl residues of the polysaccharide backbone (*i.e.* XXXG, XLXG, XXLG, and XLLG), a series consisting of longer oligosaccharide repeats was clearly observed by HPAEC-PAD

(xyloglucooligosaccharide nomenclature is according to Ref. 57), where G represents Glc*p* or β-Glc*p*-(1→4), X is [α-Xyl*p*-(1→6)]-β-Glc*p*-(1→4), and L is [β-Gal*p*-(1→2)-α-Xyl*p*-(1→6)]-β-Glc*p*-(1→4). Moreover, HPAEC-PAD and MALDI-TOF MS indicated that the ratio of the shortest oligosaccharide products deviated from the expected ratio of 1.4:1:3:5.4 XXXG/XLXG/XXLG/XLLG (58, 59). Specifically, the amount of the bis-galactosylated XLLG ($Glc_4Xyl_3Gal_2$) appeared to be reduced (supplemental Fig. S5), which suggests that extended chain branches may limit hydrolysis.

Initial Rate Kinetics on Defined Xyloglucooligosaccharides and Cellooligosaccharides—To further analyze the effect of polysaccharide branching on catalysis, we quantified the hydrolysis of well defined xyloxgluco- and cello-oligosaccharides by *Pt*EG16 under initial rate kinetic conditions. The tetradecasaccharide XXXG ↓ XXXG was exclusively hydrolyzed at the internal, unbranched β(1→4)-linked glucosyl residue (cleavage site indicated by an arrow), with a k_{cat} value of $62 \pm 6 \text{ min}^{-1}$ and a K_m value of $295 \pm 64 \text{ } \mu\text{M}$ (Fig. 4*A*). Under these conditions of low substrate conversion, transglycosylation to form $(XXXG)_3$ by disproportionation of the substrate was kinetically insignificant. However, after extended incubation of 1 mM XXXGXXXG with *Pt*EG16, minor amounts of the transglycosylation products $(XXXG)_{3-5}$ could be observed by HPAEC-PAD (supplemental Fig. S6*A*). In an independent assay, the heptasaccharide XXXG (Glc_4Xyl_3) was not hydrolyzed by *Pt*EG16-1, thus providing further support that the enzyme can only cleave the glycosidic bond of non-xylosylated glucosyl units.

In contrast to the singular mode of cleavage of XXXGXXXG by the enzyme, unbranched cello-oligosaccharides gave rise to multiple products, which complicated initial rate kinetic analysis. The longest water-soluble cello-oligosaccharide, cellohexaose, produced Glc_3 as well as pairwise equimolar amounts of Glc and Glc_5, and Glc_2 and Glc_4 (Fig. 4*B*). The Glc_2/Glc_4 hydrolysis mode dominated, with a rate approximately equal to that of XXXGXXXG hydrolysis at saturation. Summation of all cellohexaose hydrolysis modes yielded a rate approximately twice that of XXXGXXXG at saturation (Fig. 4*B*). As with XXXGXXXG, extended incubation of cellopentaose with *Pt*EG16-1 yielded apparent transglycosylation products (up to Glc_{11} in this case), as suggested by HPAEC-PAD (supplemental Fig. S6*B*).

Whereas the initial rate kinetics of cellohexaose were apparently uncomplicated by transglycosylation modes, this was not the case for cellopentaose and cellotetraose. For cellopentaose, a clear discrepancy in the rates of formation of Glc_2 and Glc_3 was observed across a range of initial substrate concentrations (supplemental Fig. S7*A*). Here, simple hydrolysis of either of the two indicated linkages, G4G4 ↓ G4 ↓ G4G4, would be expected to yield equimolar amounts of the products. The observation of a HPAEC peak with a retention time longer than cellohexaose (tentatively assigned as Glc_7, in the absence of a standard sample) was further evidence for transglycosylation.

The initial rate kinetics observed for cellotetraose were likewise complicated, including the observation of increasing production of cellohexaose with increasing substrate concentration (supplemental Fig. S7*B*). Formation of this product is
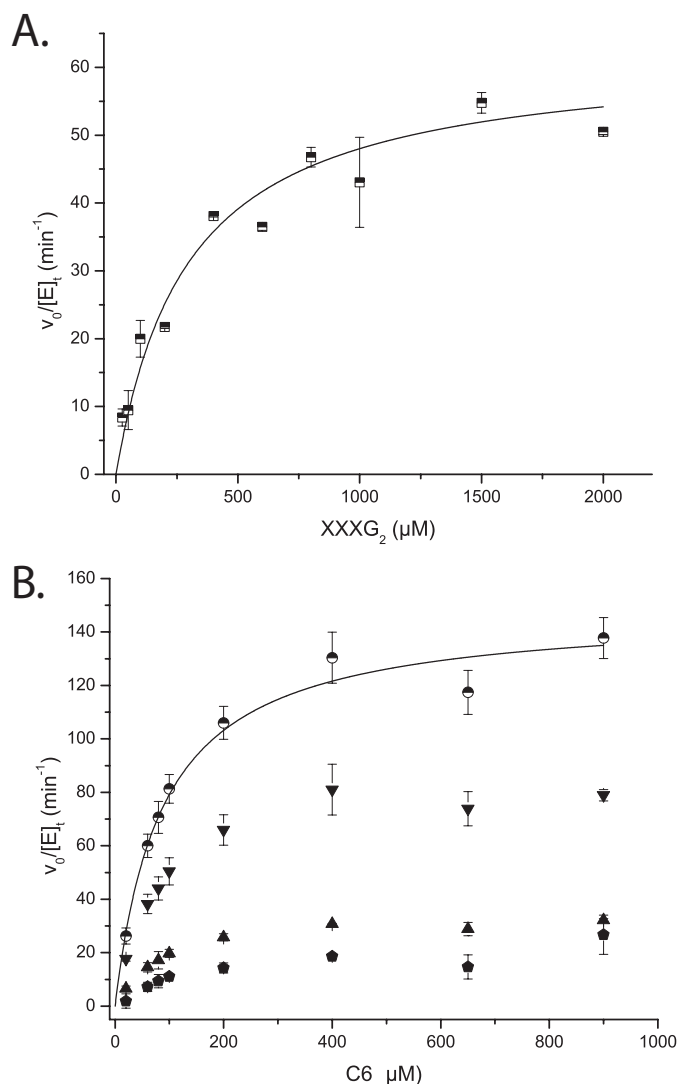
A.

B.

FIGURE 4. **Initial rate kinetic data for the *Pt*EG16-catalyzed hydrolysis of oligosaccharides.** *A*, rates of XXXG production from XXXGXXXG (XXXG$_2$). *B*, rates of production of Glc$_4$/Glc$_2$ (*inverted triangles*), Glc$_3$ (*triangles*), and Glc$_5$/Glc (*hexagons*) from cellohexaose (*C6*; Glc$_6$); *circles* represent the sum of rates for all cleavage modes. *Error bars*, S.E. from duplicate measurements. *Lines* represent the non-linear least-squares fits of the Michaelis-Menten equation to the data.

consistent with initial binding of cellotetraose in subsites -2 to $+2$, followed by cleavage to form a covalent, α-linked cellobiosyl enzyme intermediate, which is subsequently turned over by glycosyl transfer to a second molecule of cellotetraose. Of the shorter congeners, cellotriose was a very poor substrate, and cellobiose was not a substrate for *Pt*EG16 (data not shown).

Analysis of Heterotransglycosylation Potential—In light of recent interest in the capacity of certain GH16 members and crude plant enzyme extracts to catalyze xyloglucan/β-glucan heterotransglycosylation (60, 61), we examined the reaction products from two representative glycosyl donor/acceptor substrate pairs by HPAEC-PAD. For the XXXGXXXG/cellobiose pair, the product distribution was identical to that of XXXGXXXG alone (*i.e.* only longer (XXXG)$_n$ products were observed (data not shown)). In the case of cellotetraose/XXXG, repeated analysis indicated the presence of very low abundance peaks, possibly corresponding to GXXG and GGXXXG, in

addition to dominating hydrolysis and homotransglycosylation products (data not shown). However, the low amounts of these alternative products precluded isolation and definitive assignment, and we conclude that heterotransglycosylation is not kinetically significant for *Pt*EG16.

Tertiary Structure of PtEG16 and Comparison with GH16 XEHs, XETs, and Licheninases—*Pt*EG16 has thus far resisted crystallization.[3] We therefore performed *in silico* structure homology modeling, supported by experimental protein NMR spectroscopic analyses, to obtain a three-dimensional representation of the enzyme. The M4T Server version 3.0 (62) selected a bacterial GH16 licheninase (PDB code 2ayh (63)) and a plant GH16 XEH (PDB code 2uwa (11)) as templates for modeling full-length *Pt*EG16. For comparison, we also subjected *Pt*EG16 to structural modeling using Protein Homology/Analogy Recognition Engine version 2.0 (Phyre2 (64)). Both approaches produced tertiary structures with a β-jellyroll fold typical of GH16 enzymes. Moreover, superimposition of the Cα traces indicated that these predicted structures were nearly identical (Fig. 5*A*; root mean square deviation values are given in supplemental Table S3).

To provide experimental validation for the models, we investigated the recombinant *Pt*EG16 using NMR spectroscopy. Under reducing conditions, the protein yielded an excellent quality, well dispersed ^{15}N TROSY-HSQC spectrum with peak line widths indicative of a stably folded, monomeric protein (Fig. 5*B*). In total, of the 213 residues (not including the His$_6$ tag), main chain ^{1}H^N, ^{15}N, ^{13}C′, ^{13}C$^\alpha$, and ^{13}C$^\beta$ signals from 166 were unambiguously assigned via a manual analysis of standard triple resonance correlation experiments. An additional 14 spin systems were assigned using the PINE assignment server (65). Chemical shift assignments for only the ^{13}C′, ^{13}C$^\alpha$, and ^{13}C$^\beta$ nuclei were obtained for a further 29 residues (including 8 of the 11 prolines). Four residues were left without any assigned resonances (Pro-22, Pro-163, Gly-183, and Ser-213). The non-proline residues that are without amide proton and nitrogen assignments are located throughout the protein (supplemental Fig. S8). The lack of assignments may reflect spectral overlap, rapid amide hydrogen exchange, or conformational exchange broadening.

The NMR chemical shifts of main chain nuclei are sensitive indicators of protein secondary structure (66, 67). We therefore used the SPP (68), MICS (41), and TALOS+ (42) algorithms to determine the secondary structural elements of *Pt*EG16 from the assigned chemical shifts. All three algorithms yielded similar results, identifying numerous β-strands with a limited number of short helical regions. Importantly, these secondary structural elements, derived from experimental data, agreed well with those present in the M4T structural model, as defined by VADAR (69) (supplemental Fig. S8).

Further experimental validation of the model was obtained by calculating the overall *Pt*EG16 fold from backbone chemical shift data using the CS23D Web server (70). The resulting
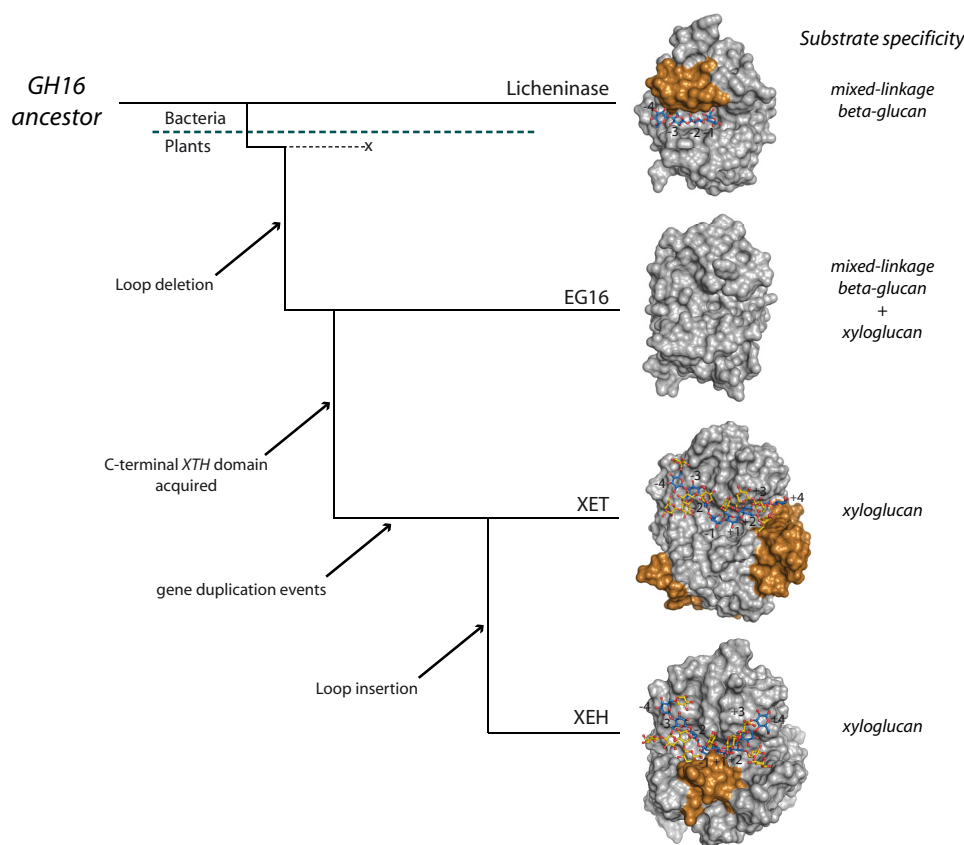
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[3] M. Czjzek, unpublished data.

FIGURE 5. **Modeling and experimental validation of the PtEG16 tertiary structure.** *A*, superimposition of the M4T (*marine*) and Phyre2 (*magenta*) homology models and the chemical shift-derived CS23D (*orange*) model of *Pt*EG16 in a *wall-eyed stereo* representation. Models are oriented with the positive enzyme subsites on the *right*, relative to the conserved active-site residues Glu-88, Asp-90, and Glu-92 (*bottom* to *top*) of *Pt*EG16 (*cf*. Fig. 1). *B*, assigned ^{15}N TROSY-HSQC spectrum of carbon-perdeuterated and ^{13}C,^{15}N-labeled *Pt*EG16 in H$_2$O buffer at pH 7.5 and 25 °C.

FIGURE 6. **Proposed evolution of *EG16* and *XTH* gene products in GH16 from a licheninase-like ancestor.** With reference to Fig. 1, the *gold coloring* represents the licheninase loop extension, the XET C-terminal extension, and the XEH YNIIG loop insertion in the respective proteins.

model superimposes very well the homology models from M4T and Phyre2 (Fig. 5*A* and supplemental Table S3). Moreover, superimposition with the M4T templates (licheninase (PDB code 2ayh) and XEH (PDB code 2uwa)), indicate that the *Pt*EG16 models possess the canonical GH16 fold with a broader active-site cleft, which is typical of plant *XTH* gene products (supplemental Fig. S9 and Table S3; *cf.* Fig. 6).

Inspection of these superimposed models indicates that this broadening of the active-site cleft has resulted from the deletion of a ~12-amino acid sequence found in the bacterial licheninases (Fig. 1, residues 23–34). This loop extends into the concave face of the β-jellyroll to form a part of the negatively numbered enzyme subsites (supplemental Fig. S9*A*), thereby narrowing the active-site cleft (Fig. 6) and contributing many structural features necessary for specific recognition of kinked mixed linkage glucan chains (16, 48, 71). The removal of this loop in plant *Pt*EG16 and *XTH* gene products is a clear prerequisite for the binding of highly branched xyloglucans (72–74).

In the positively numbered subsites, *Pt*EG16 resembles a licheninase, primarily because it lacks the C-terminal extension characteristic of true *XTH* gene products (supplemental Fig. S9*B*). In XETs and XEHs, this motif elongates the substrate binding cleft by providing one β-sheet, narrows the positive subsites (Fig. 6), and supplies two-thirds of a Xyl +2′ xyloglucan specificity pocket that affects specificity (k_{cat}/K_m) 500-fold (72, 75). Like the licheninases, *Pt*EG16 also lacks the small loop insertion immediately following the catalytic motif (NRT in *Ptt*XET16-34; Fig. 1), which further affects the structure of the

positively numbered subsites in *XTH* gene products and also contains the conserved *N*-glycosylation site important for the stability of *XTH* Group I/II members (76, 77).

DISCUSSION

Barbeyron and colleagues (15, 78) were among the first to speculate upon the evolutionary basis of the diverse catalytic functionality observed among GH16 members. Although enzyme structure-function analyses continue to shed light on protein loop differences that fine-tune specificity for particular linear galactans and glucans among bacterial members (15, 79, 80), the evolutionary steps leading to the dramatic structural features of plant *XTH* gene products have remained essentially unknown.

Taken together, our present data suggest that a unique protein in black cottonwood, *Pt*EG16, is a broad specificity glycoside hydrolase with nearly equal capacity to cleave linear glucans (mixed linkage β-glucans and cello-oligosaccharides) and highly branched xyloglucan oligo- and polysaccharides (*e.g.* Figs. 3 and 4). Moreover, this broad specificity appears to arise from a protein scaffold that is intermediate between extant bacterial licheninases and plant *XTH* gene products (Figs. 2 and 5). As such, these observations allow us to posit that *Pt*EG16-like sequences represent an ancestral link in the evolution of extant plant XETs and XEHs.

Fig. 6 highlights a view of this potential evolution from the perspective of protein tertiary structure, supported by sequence-based phylogeny (Fig. 2*A*). In this scheme, an early

Broad Specificity P. trichocarpa GH16 Endo-β-glucanase

GH16 ancestor with a structure similar to extant licheninases may have first given rise to a *Pt*EG16-like protein via deletion of the licheninase-specific negative subsite loop. Although we cannot rigorously exclude the possibility that a *Pt*EG16-like protein was the ancestor and that licheninases arose by loop addition, the apparent lack of direct *Pt*EG16 homologs in the "lower" organisms, bacteria in particular, suggests otherwise.

The lack of this loop opened the active-site cleft and expanded the potential substrate range of *Pt*EG16 homologs (Fig. 6). The observation of *Pt*EG16 activity on both mixed linkage β-glucans and xyloglucan is particularly noteworthy, because these are the dominant matrix polysaccharides in grassy monocots and dicots, respectively. *Pt*EG16 homologs are present in early plant lineages (Fig. 2*B*), and this dual activity would have thus poised them for subsequent functional adaptation in emerging species (6, 81) that favored either type of polysaccharide as a wall component. Single *Pt*EG16 homologs are indeed found in many monocots and dicots (Fig. 2*B*). Interestingly, *Pt*EG16-like genes, in particular in early diverging plants and monocots, have simple structures that lack introns, which may be further evidence of their bacterial origins (supplemental Table S2) (82).

Subsequently in the proposed evolution (Fig. 6), an early *Pt*EG16 homolog is suggested to have acquired the large *XTH* C-terminal extension (Pfam *XET_C*, PF06955; Fig. 1), which is unique among GH16 members. This extension is found in all true *XTH* gene products from all major phylogenetic clades (Group I/II, III-A, and III-B; Fig. 2*B*), including all biochemically characterized XETs and XEHs (13). This suggests that acquisition of the C-terminal extension predated the massive expansion of *XTH* genes in mosses and later-diverging plants (Fig. 2*B*) (*e.g.* see Refs. 8, 9, and 82). The genetic mechanism of this acquisition is currently unknown.

Although systematic studies are lacking for most *XTH* gene products (13), it appears that at least some have lost much or all of their putative ancestors' ability to act upon mixed linkage β-glucans. Specifically, barley *Hv*XET5 acts on barley mixed linkage β-glucan at ≤0.2% of its rate on xyloglucan, whereas nasturtium *Tm*NXG1 (an XEH associated with seed storage polysaccharide hydrolysis) has no detectable activity on Icelandic moss lichenan or barley β-glucan (11). There is no *a priori* reason why XETs or XEHs should exclude unbranched β-glucans from their active-site clefts based on steric considerations. However, specific enzyme-substrate interactions have apparently been optimized to favor xyloglucan binding in these *XTH* gene products (11, 45, 72, 73, 75).

Finally, we have previously provided evidence that the comparatively small clade of Group III-A *XTH* gene products (Fig. 2*A*), which comprises predominant XEHs, arose from the larger body of XETs via the specific acquisition of two small loop insertions altering the active-site cleft topology (11, 13) (Fig. 6; *cf.* Fig. 1). Combined phylogenetic, biochemical, and tertiary structural analyses indicated that a 5-amino acid loop (YNIIG; Fig. 1) is a primary factor contributing to the predominant hydrolytic (XEH) capacity of nasturtium *Tm*NXG1. Deletion of this loop significantly increased the transglycosylation/hydrolysis ratio of the enzyme, making it structurally and functionally

more XET-like (11). This loop insertion is likewise found in two homologous *A. thaliana* XEHs of Group III-A (12).

In summary, the evolutionary scheme proposed here provides a new insight into the origins of the *XTH* gene products and a framework for the further exploration of protein structure-function relationships in plant GH16 members. Many questions remain outstanding, especially for *Pt*EG16 homologs. These include the following.

To What Extent Can the Endoglucanase Designation Be Reliably Extended to Other PtEG16 Homologs?—We cautiously suggest that all members of the closely related dicot and monocot EG16-like clades may bear the same general activity profile as *Pt*EG16 (Fig. 2*A*). However, the lack of demonstrable XET, XEH, or licheninase activity in one of three *P. patens* homologs (XTH32 (82)) suggests that careful functional analysis of early-diverging plant (moss) clades may especially be warranted.

What Is the in Vivo Role(s) of PtEG16 Homologs across Plant Lineages?—Although a comprehensive transcriptomic analysis has not been performed for any species, the gene encoding *Pt*EG16 (previously known as *XTH8*) appears to be hormonally regulated (50). Likewise, a *Pp*XTH32 promotor-glucuronidase fusion indicates tissue-specific expression in *P. patens*. The presence of single homologs in most species should facilitate reverse genetics analyses. Moreover, the lack of multiplication of *Pt*EG16 homologs in plant genomes on a par with *XTH* genes may indicate either that they are functionally unique and under tight control or that they are simply molecular "transitional fossils," vestigial sequences with no significant function. The observation that *Pt*EG16 homologs are missing in many dicots might suggest the latter (Fig. 2*A*), although their functional importance may be inversely correlated with plant evolution. The lack of obvious signal peptides in *Pt*EG16 homologs and a high abundance of Cys residues in some proteins (*e.g. Pt*EG16), which may suggest intracellular localization, is particularly puzzling and relevant to this question.

What Is the Distribution and Function of PtEG16 and XTH Homologs in the Genomes of the Charophycean Green Algae?—Some members of this diverse group are the most primitive organisms known to have plantlike cell walls, including hemicellulosic polysaccharides like β(1→3)-glucans and xyloglucans (81). In notable contrast, the chlorophycean green algae do not possess xyloglucan (81) and, correspondingly, do not have any *XTH*-like genes (13) (Fig. 2*B*). We anticipate that forthcoming genomes of charophytes will illuminate the early origins of *Pt*EG16 and *XTH* genes and their evolutionary relatedness.

In This Context, What Protein Structural Features Are Responsible for Substrate Specificity and Biochemical Function of PtEG16-like Proteins?—Our present homology models only allow reliable analysis of gross structural features on the level of overall fold and active site topology (Fig. 6). High resolution structural enzymology on par with that for other GH16 enzymes will be required to illuminate the details of substrate recognition and catalysis, which underpin biochemical and physiological function.

CONCLUSION

In harness, phylogenetic, biochemical, and protein structural analysis have revealed a unique clade of plant proteins in glyco-

side hydrolase family 16, whose members may represent a key step in the evolution of the widespread and diverse *XTH* gene family in plants. As such, the revelation of this clade will help refine future bioinformatics analyses of plant genomes. Moreover, further structural and functional analysis will undoubtedly bring new insight into the roles of individual clade members in the context of plant physiology.

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